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(54) Title: ANTISENSE OLIGONUCLEOTIDES SPECIFIC FOR THYMIDYLATE SYNTHASE

## (57) Abstract

The present invention discloses synthetic oligonucleotides complementary to thymidylate synthase and methods of downregulating at least a portion of thymidylate synthase mRNA translation or protein expression in a malignant cell and of preventing or overcoming resistance to thymidylate synthase inhibitor compounds in a malignant cell using such oligonucleotides. Also disclosed is a therapeutic composition and a method of treating malignant tumor growth in a mammal utilizing such a composition.

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## ANTISENSE OLIGONUCLEOTIDES SPECIFIC FOR THYMIDYLATE SYNTHASE

BACKGROUND OF THE INVENTION:

Thymidylate synthase (TS), an important target in cancer chemotherapy, is critical for the intracellular *de novo* synthesis of thymidylate, a necessary precursor for DNA biosynthesis. Studies have shown that malignant cells have a higher requirement for precursors of DNA biosynthesis, thus it is possible to target a malignant cell's ability to produce TS protein while maintaining viability of normal cells. However, studies have shown that the levels of TS protein are acutely induced following exposure of human breast and colon cancer cells to the antimetabolite, 5-fluorouracil (5-FU). Elevation of TS protein was also observed in tumor specimens from patients treated with 5-FU suggesting that this process is clinically relevant. Chu et al. *Proc Natl Acad Sci. USA* **88**:8977-8981 (1991) was the first group to demonstrate that the expression of TS is controlled by a novel translational autoregulatory feed-back mechanism whereby the protein binds to its own TS mRNA. Under normal conditions, this RNA/protein interaction results in inhibition of TS protein synthesis. Upon treatment with inhibitor compounds of TS such as 5-FU, this normal RNA/protein intercation is abrogated, allowing new synthesis of TS protein. Chu et al., *Cancer Res.* **50** 5834-5840 (1990); Chu et al, *Proc Natl Acad. Sci USA* **90**:517-521 (1993); Chu et al., *Mol Pharmacol.* **43**: 4756-4760 (1993).

New approaches to prevent and/or overcome resistance to TS inhibitor compounds are needed, as are compounds and methods that specifically repress TS mRNA translation directly, resulting in growth inhibition of malignant tumors.

### SUMMARY OF THE INVENTION

It has been discovered that oligonucleotides directed to TS mRNA can inhibit the expression of the TS protein, and thereby can prevent or overcome resistance to TS inhibitor compounds as well as cause growth inhibition of cancerous tumors.

As used herein, the term "oligonucleotide" is meant to include polymers of two or more nucleotides or nucleotide analogs connected together via 5' to 3' internucleotide linkages which may include any linkages that are known in the antisense art. Such molecules have a 3' terminus and a 5' terminus. The term "synthetic oligonucleotide" refers to oligonucleotides synthesized by other than natural processes, such as by biochemical or genetic engineering methods.

As used herein, the term "a TS nucleic acid" refers to genomic DNA and transcript thereof, cDNA, mRNA, and pre- mRNA which encode the TS protein, or portions thereof, 3' or 5' untranslated regions or other regulatory regions, introns, or splice junction sites. The term "complementary to" refers herein to oligonucleotides which are capable of hybridizing or otherwise associating with at least a portion of such a TS nucleic acid under physiological conditions.

In some embodiments, synthetic oligonucleotides of the invention are complementary to a portion of TS nucleic acid which encodes the 5' untranslated region, the 3' untranslated region, the translational start site, the translational stop site, or a splice junction site. As used herein, the term "splice junction site" is meant to encompass the splice donor, splice acceptor, or intron-exon boundary regions of the nucleic acid.

In some preferred embodiments, the synthetic oligonucleotides of the invention consist essentially of nucleic acids sequences having the nucleotide sequence 5'-AGC CGG CCA CAG GCA TGG CGC GGC GGG CGG-3' (SEQ ID NO:1), 5'AGC CGG CCA CAG GCA UGG CGC GGC GGG CGG-3' (SEQ ID NO: 2), and modifications and derivatives thereof. Some oligonucleotides of the invention have from about 6 to about 50 nucleic acids. Other oligonucleotides of the invention have from about 15 to about 30 nucleic acids. Yet other oligonucleotide have from 20 to 25 nucleic acids.

In some embodiments of the invention, the oligonucleotides administered are modified with other than phosphodiester-internucleotide linkages between the 5' end of one nucleotide and the 3' end of another nucleotide, in which the 5' nucleotide phosphate has been replaced with any number of chemical groups.

As used herein, the term "modified oligonucleotide" encompasses oligonucleotides with at least one non- phosphodiester internucleotide linkage, modified nucleic acid(s), base(s), and/or sugar(s) other than those found in nature. For example, a 3', 5'-substituted oligonucleotide is an oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

A modified oligonucleotide may also be one with added substituents such as diamines, cholestryl, cholesterol, or other lipophilic groups, or a capped species. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides. Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found in vivo without human intervention are also considered herein as modified.

In some embodiments of the invention, the oligonucleotide has at least one modified internucleotide linkage such as an alkylphosphonate, phosphorothioate, phosphorodithioate, alkylphosphonothioate, alkylphosphonate, phosphoramidate, phosphate ester, carbamate, acetamide, carboxymethyl ester, carbonate, or phosphate triesters. In some preferred embodiments, the oligonucleotide has phosphorothioate internucleotide linkages.

Oligonucleotides of the invention may include one ribonucleotide which is a 2'-O-substituted ribonucleotide. For purposes of the invention, the term "2'-substituted oligonucleotide" refers to an oligonucleotide having a sugar attached to a chemical group other than a hydroxyl group at its 2' position. The 2'-OH of the ribose molecule can be substituted with -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms, e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl (such as a 2'-O-methyl), 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. In some embodiments, the ribonucleotide is a 2'-O-alkylated ribonucleotide such as a 2'-O-methylated ribonucleotide.

In one preferred embodiment the oligonucleotide is composed of 2'-substituted ribonucleotides in all positions, preferably 2'OMe, and furthermore, comprises one or more modified internucleotide linkages, such as phosphorothioate internucleotide linkages.

In another aspect, the present invention provides a method of preventing or overcoming resistance to TS inhibitor compounds in a malignant cell, comprising the step of administering to the cell an amount of at least one oligonucleotide of the invention sufficient to downregulate at least a portion of TS mRNA translation or TS protein expression in an amount sufficient to overcome resistance to TS inhibitor compounds in the cell.

The invention also provides a method of downregulating at least a portion of the synthesis of TS protein in a malignant cell comprising the step of administering to the cell an amount of an oligonucleotide of the invention sufficient to specifically repress at least a portion of TS mRNA translation and downregulate the synthesis of protein in the cell. As used herein "downregulation of at least a portion of TS mRNA translation or TS protein expression" refers to the reduction or elimination of a malignant cell's ability to express the TS protein.

Another aspect of the invention is a method of inhibiting the growth of a cancerous cell which has become resistant to TS inhibitor compounds, comprising the step of administering to the cell an amount of an oligonucleotide of the invention sufficient to slow or stop growth of the cell and optionally, simultaneously or sequentially administering a TS inhibitor compound.

Yet another aspect is a therapeutic composition comprising at least one TS-specific oligonucleotide of the invention and a pharmaceutically acceptable carrier or diluent. As used herein, a "pharmaceutically or physiologically acceptable carrier or diluent" includes any and all solvents (including but not limited to lactose), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. Optionally, a therapeutic composition of the invention may include a TS inhibitor compound. As used herein a TS inhibitor compound includes but is not limited to 5-fluorouracil (5-FU) and antifolate analogs.

Also provided by the present invention are methods of treating cancer in a mammal and of preventing or overcoming resistance to TS inhibitor chemotherapy in a mammal afflicted with cancer. In this method, a therapeutically effective amount of the therapeutic formulation of the invention is administered to the mammal. Optionally, the mammal may be pre-treated or treated simultaneously or sequentially with a TS inhibitor compound.

For purposes of the invention, the term "mammal" is meant to encompass primates and humans. The term "therapeutically effective amount" refers to the total amount of each active component of the pharmaceutical formulation or method that is sufficient to show a meaningful subject or patient benefit, i.e., healing of disease conditions characterized by the disease being treated, an increase in rate of healing of such conditions, a reduction in the rate of cell or tumor growth, and a reduction in TS activity via reduced or altered levels of expression of TS or cells which cause or characterize the disease or disorder being treated.

The subject oligonucleotides and methods of the invention also provide a means of examining the function of the TS gene in a cell, or in a control mammal and in a mammal afflicted with cancer. The cell or mammal is administered the oligonucleotide, and the expression of TS protein and/or proteins which are known to interact with TS is examined. Presently, gene function is often examined by the arduous task of making a "knock out" animal such as a mouse. This task is difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock out" would produce a lethal phenotype. The present invention overcomes the shortcomings of this model.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the specific inhibition of TS mRNA by a 2'OH antisense RNA oligo *in vitro*.

Fig 2 shows translational inhibition of TS mRNA by various antisense oligonucleotides.

Fig. 3 shows inhibition of MCF-7 cell growth by antisense HYB0432.

Fig. 4 shows a Western analysis of TS protein in MCF-7 cells after treatment with HYB0432.

Fig 5 shows the complete human TS cDNA sequence. Nucleotides are numbered in the 5'-3' direction. Position 1 corresponds to the first nucleotide of the ATG triplet. The predicted amino acid sequence of human TS is shown in italics with asterisks at the stop codon. The amino acids residues are numbered beginning with the initiator methionine.

#### DETAILED DESCRIPTION OF THE DRAWING

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patent, allowed patent applications, and articles cited herein are hereby incorporated by reference.

It is known that antisense oligonucleotides, called an "antisense oligonucleotide," can bind to a target single-stranded nucleic acid molecule according to the Watson-Crick, or the Hoogsteen rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of mRNA by RNase H if a contiguous region of deoxyribonucleotides exists in the oligonucleotide, and/or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Oligonucleotides of the invention have a nucleotide sequence which is complementary to a TS-specific nucleic acid sequence. Such oligonucleotides can be directed to any portion of a TS nucleic acid, as the genomic sequence is known as is the cDNA sequence (Takeishi et al, *Nucleic Acids Research* 13:2035-2042 (1985)). One preferable region to which TS-specific oligonucleotides of the invention spans the 5' untranslated region and the translational start region. This region is believed to be a

critical cis-acting regulatory element wherein the TS mRNA is bound by its own protein end product, TS, in a negative autoregulatory manner. Other preferred regions to which oligonucleotides of the invention are directed include, but are not limited to, the 3'UTR region, the translational stop site and splice junction sites.

The oligonucleotides of the invention are at least 6 nucleotides in length, but are preferably 6 to 50 nucleotides long, with 15 to 30mers being common, and 20mers to 25mers being the most common.

Oligonucleotides directed to the translational start or stop site preferably include the three nucleotides complementary to the start or stop site codon and at least 3, preferably at least 7, and more preferably, at least 17 additional nucleotides. Oligonucleotides directed to the splice junction sites are complementary to at least three nucleotides of the splice donor, splice acceptor, or intron/exon boundary.

Table 1 lists some nonlimiting representative species of oligonucleotides which are useful in the method of the invention.

TABLE 1

Target	Region	Sequence	SEQ ID NO
80-109	Tr. Start	agccggccacaggcatggcgccggcgccgg	1
80-109	Tr. Start	agccggccacaggcauggcgccggcgccgg	2

*Tr Start-translational start region*

With the published nucleic acid sequences and this disclosure provided, those of skill in the art will be able to identify, without undue experimentation, other antisense nucleic acid sequences that inhibit TS expression. For example, other sequences targeted

specifically to human TS nucleic acid can be selected based on their ability to inhibit translation of TS mRNA and/or inhibit TS protein expression.

For purposes of the invention, the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence that binds to the target nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means including in the case of a oligonucleotide binding to RNA, pseudoknot formation. Such binding (by Watson Crick base pairing) under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

Thus, because of the properties described above, such oligonucleotides are useful therapeutically because of their ability to control or down-regulate the expression of the TS gene in a mammal, according to the method of the present invention.

They are composed of deoxyribonucleotides, ribonucleotides, or a combination of both, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked by non-phosphodiester internucleotide linkages. Such linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. Preferably, the oligonucleotides of the invention are linked with at least one phosphorothioate internucleotide linkage. Other preferred oligonucleotides of the invention have at least two different internucleotide linkages within the same molecule.

For example, U.S. Patent No. 5,149,797 describes traditional chimeric oligonucleotides having a phosphorothioate core region interposed between methylphosphonate or phosphoramidate flanking regions. U.S. Patent Application Ser. No. 08/516,454, filed on August 9, 1995 discloses "inverted" chimeric oligonucleotides comprising one or more nonionic oligonucleotide region (e.g. alkylphosphonate and/or phosphoramidate and/or phosphotriester internucleoside linkage) flanked by one or more region of oligonucleotide phosphorothioate. Oligonucleotides with these linkages can be prepared according to known methods such as phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as described

by Brown (*A Brief History of Oligonucleotide Synthesis. Protocols for Oligonucleotides and Analogs. Methods in Molecular Biology* (1994) 20:1-8). (See also, e.g., Sonveaux "Protecting Groups in Oligonucleotides Synthesis" in Agrawal (1994) *Meth. Mol. Biol.* 26:1-72; Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

The oligonucleotides of the composition may also be modified in a number of other ways without compromising their ability to hybridize to the target nucleic acid. Such modifications include, for example, those which are internal or at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule. For the preparation of such modified oligonucleotides, see, e.g., Agrawal (1994) *Methods in Molecular Biology* 26; Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

Oligonucleotides which are self-stabilized are also considered to be modified oligonucleotides useful in the methods of the invention (Tang et al. (1993) *Nucleic Acid Res.* 20:2729-2735). These oligonucleotides comprise two regions: a target hybridizing region; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

The preparation of these unmodified and modified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158) (see, e.g., Uhlmann et al. (1990) *Chem. Rev.* 90:543-584; and (1987) *Tetrahedron Lett.* 28:(31):3539-3542); Agrawal (1994) *Methods in Molecular Biology* 20:63-80); and Zhang et al. (1996) *J. Pharmacol. Expt. Thera.* 278:1-5.

The oligonucleotides administered to the animal may be hybrid oligonucleotides in that they contain both deoxyribonucleotides and at least one 2' substituted ribonucleotide. For purposes of the invention, the term "2'- substituted" means substitution at the 2' position of the ribose with, e.g., a -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. Useful substituted ribonucleotides are 2'-O-alkyls such as 2'-O-methyl.

The hybrid DNA/RNA oligonucleotides useful in the method of the invention resist nucleolytic degradation, form stable duplexes with RNA or DNA, and preferably activate RNase H when hybridized with RNA. They may additionally include at least one unsubstituted ribonucleotide. For example, an oligonucleotide of the invention may contain all deoxyribonucleotides with the exception of at least one or at least two 2'-substituted ribonucleotides at the 3'-terminus or the 5'- terminus of the oligonucleotide. Alternatively, the oligonucleotide may have at least one or at least two substituted ribonucleotide at both its 3' and 5' termini.

One preferred class of oligonucleotides of the invention are antisense oligonucleotides which do not activate RNase H. Such antisense oligonucleotides, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the chemical moieties involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense oligonucleotides which do not activate RNase H are available. For example, such antisense oligonucleotides may be oligonucleotides wherein at least one or all of the internucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphonothiates, phosphoromorpholidates, phosphoropeperazidates, and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described.

In one preferred embodiment, such antisense oligonucleotides are oligonucleotides wherein at least one, or all, of the nucleotides contain a 2' loweralkyl moiety (e.g., C1-C4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, all of the nucleotides of an antisense molecule may contain 2'-OMe substituted RNA, or just some of the oligonucleotides (e.g., every other, or every third etc of the nucleotides may be so modified). See also P. Furdon et al., *Nucleic Acids Res.* **17**, 9193-9204 (1989); S. Agrawal et al., *Proc Natl. Acad. Sci. USA* **87**, 1401-1405 (1990); C. Baker et al., *Nucleic Acids Res.* **18**, 3537-3543 (1990); B. Sproat et al., *Nucleic Acids Res.* **17**, 3373-3386 (1989); R. Walder and J. Walder, *Proc. Natl. Acad. Sci. USA* **85**, 5011-5015 (1988).

In one preferred embodiment of the invention, the oligonucleotide is a ribonucleotide. In another preferred embodiment of the invention, the oligonucleotide is an all 2'OMe substituted ribonucleotide. In yet another preferred embodiment of the invention, the oligonucleotide is an all 2'-OMe substituted ribonucleotide wherein all internucleotide linkages are phosphorothioate (PS) internucleotide linkages.

Some non-limiting representative preferred oligonucleotides of the invention are shown in Table 2 below:

TABLE 2

Oligo	Target	Sequence (5'-3')	Seq ID No
DNA-AS	80-109	agccggccacaggcatggcgccggcggcgg	1
2'OH RNA AS	80-109	<i>agccggccacaggcauggcgccggcggcgg</i>	2
HYB0432	80-109	<b>agccggccacaggcauggcgccggcggcgg</b>	2
HYB0431	80-109	<u>agccggccacaggcauggcgccggcggcgg</u>	2

*italics* represent 2'-OH ribonucleotides

**bold** represents 2'-OMe ribonucleotides

underline represents phosphorothioate internucleotide linkages

all other internucleotide linkages are phosphodiester unless indicated otherwise.

The oligonucleotides according to the invention are effective in inhibiting at least a portion of the expression of TS, and particularly in inhibiting at least a portion of protein expression, in cells *in vivo* or *in vitro*. The ability to inhibit at least a portion of

the expression of the TS protein is clearly important to the treatment of a variety of malignant cancers.

One aspect of the invention provides therapeutic compositions suitable for treating malignant cancers. Such a therapeutic composition includes at least one TS-specific oligonucleotide of the invention and a pharmaceutically or physiologically acceptable carrier or diluent. As used herein, a "pharmaceutically or physiologically acceptable carrier or diluent" includes any and all solvents (including but not limited to lactose), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions of the invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions. A therapeutic composition may optionally include a TS inhibitor compound.

In one preferred therapeutic composition of the invention, about 25 to 75 mg of a lyophilized oligonucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NOS:1-2 and 20-75 mg lactose, USP, which is reconstituted with sterile normal saline to the therapeutically effective dosages described herein. Another preferred therapeutic composition of the invention comprises about 50 mg of an oligonucleotide having SEQ ID NO:1 or 2 and about 40 mg lactose.

Another aspect of the invention provides methods for treating mammals suffering from malignant cancer which has become resistant to TS inhibitor compounds, or the overexpression of the TS gene. In this method a therapeutically effective amount of a therapeutic composition of the invention is administered to the mammal. Such methods of treatment according to the invention, may be administered in conjunction with other anticancer therapeutic agents or treatments, e.g., TS inhibitors such as 5-FU.

As used herein, the term "therapeutically effective amount" refers to the total amount of each active component of the pharmaceutical formulation or method that is sufficient to show a meaningful subject or patient benefit, i.e., a reduction in or arrest of the growth rate of the tumor or a reduction in the size of the cancer or tumor; healing of disease conditions characterized by the particular disorder being treated and/or an increase in rate of healing of such conditions; and a reduction in the rate of expression of

which directly or indirectly cause or characterize the disease or disorder being treated. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

A "therapeutically effective manner" refers to a route, duration, and frequency of administration of the pharmaceutical formulation which ultimately results in meaningful patient benefit, as described above. In some embodiments of the invention, the pharmaceutical formulation is administered via injection, sublingually, rectally, intradermally, orally, or enterally in bolus, continuous, intermittent, or continuous, followed by intermittent regimens.

The therapeutically effective amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the dosages of the pharmaceutical compositions administered in the method of the present invention should contain about 10  $\mu$ g to about 20 mg of synthetic oligonucleotide per kg body or organ weight, preferably 0.1 to 5.0 mg/kg body weight per day, and more preferably 0.1 to 2.0 mg/kg body weight per day. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of oligonucleotide from about 0.01  $\mu$ M to about 10  $\mu$ M. Preferably, the concentration of oligonucleotide at the site of aberrant gene expression should be from about 0.01  $\mu$ M to about 10  $\mu$ M, and most preferably from about 0.05  $\mu$ M to about 5  $\mu$ M. However, for localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. It may be desirable to administer simultaneously or sequentially a therapeutically effective amount of one or more of the therapeutic compositions of the invention when individual as a single treatment episode.

Administration of pharmaceutical compositions in accordance with invention or to practice the method of the present invention can be carried out in a variety of conventional ways, such as by oral ingestion, enteral, rectal, or transdermal administration, inhalation, sublingual administration, or cutaneous, subcutaneous, intramuscular, intraocular, intraperitoneal, or intravenous injection, or any other route of administration known in the art for administrating therapeutic agents.

When the composition is to be administered orally, sublingually, or by any non-injectable route, the therapeutic formulation will preferably include a physiologically acceptable carrier, such as an inert diluent or an assimilable edible carrier with which the composition is administered. Suitable formulations that include pharmaceutically acceptable excipients for introducing compounds to the bloodstream by other than injection routes can be found in Remington's Pharmaceutical Sciences (18th ed.) (Genarro, ed. (1990) Mack Publishing Co., Easton, PA). The oligonucleotide and other ingredients may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. The therapeutic compositions may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. When the therapeutic composition is administered orally, it may be mixed with other food forms and pharmaceutically acceptable flavor enhancers. When the therapeutic composition is administered enterally, they may be introduced in a solid, semi-solid, suspension, or emulsion form and may be compounded with any number of well-known, pharmaceutically acceptable additives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated such as those described in U.S. Patent Nos. 4,704,295, 4,556,552, 4,309,404, and 4,309,406.

When a therapeutically effective amount of a composition of the invention is administered by injection, the synthetic oligonucleotide will preferably be in the form of a pyrogen-free, parenterally-acceptable, aqueous solution. The preparation of such parenterally-acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms, such as bacterial and fungi. The carrier can be a solvent or dispersion medium. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents. Prolonged absorption of the injectable therapeutic agents can be brought about by the use of the compositions of agents delaying absorption. Sterile injectable solutions are prepared by incorporating the oligonucleotide in the required amount in the appropriate solvent, followed by filtered sterilization.

The pharmaceutical formulation can be administered in bolus, continuous, or intermittent dosages, or in a combination of continuous and intermittent dosages, as determined by the physician and the degree and/or stage of illness of the patient. The duration of therapy using the pharmaceutical composition of the present invention will vary, depending on the unique characteristics of the oligonucleotide and the particular therapeutic effect to be achieved, the limitations inherent in the art of preparing such a therapeutic formulation for the treatment of humans, the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

The therapeutic pharmaceutical formulation containing the oligonucleotide includes a physiologically acceptable carrier, such as an inert diluent or an assimilable edible carrier with which the peptide is administered. Suitable formulations that include pharmaceutically acceptable excipients for introducing compounds to the bloodstream by other than injection routes can be found in Remington's Pharmaceutical Sciences (18th ed.) (Genarro, ed. (1990) Mack Publishing Co., Easton, PA). The oligonucleotide and other ingredients may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. The oligonucleotide may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. When the oligonucleotide is administered orally, it may be mixed with other food forms and pharmaceutically acceptable flavor enhancers. When the oligonucleotide is administered

enterally, they may be introduced in a solid, semi-solid, suspension, or emulsion form and may be compounded with any number of well-known, pharmaceutically acceptable additives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated such as those described in U.S. Patent Nos. 4,704,295, 4,556,552, 4,309,404, and 4,309,406.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount since the necessary effective amount can be reached by administration of a plurality of dosage units (such as capsules or tablets or combinations thereof).

In order to determine whether antisense oligonucleotides of the invention complementary to TS nucleic acid are able to specifically repress at least a portion of the TS mRNA translation, *in vitro* translation studies were conducted using a rabbit reticulocyte lysate translation system. In a first study the specific translational inhibition of TS mRNA by a 2'OH antisense RNA oligonucleotide was tested. Human TS mRNA (100 ng), *E. coli* TS mRNA (300ng) and luciferase mRNA (400 ng) along with various amounts of 2'OH RNA-AS (SEQ ID NO: 2) were added to rabbit reticulocyte lysate (RRL) reactions. The signal intensities from densitometry scanning were normalized against the signal intensity for each mRNA in the absence of antisense oligonucleotides. The results in Fig. 1 show that 2'OH RNA-AS (SEQ ID NO: 2) significantly repressed human TS mRNA translation in a specific manner (i.e. the control mRNAs from *E. coli* and luciferase were not inhibited).

In a second study, translation inhibition of human TS mRNA by various antisense oligoribonucleotides of the invention was determined. Both TS mRNA (100ng) and antisense oligos were incubated in rabbit reticulocyte lysate reactions. The results are shown in Fig. 2 where each point represents the mean  $\pm$  S.D. from 2-4 separate experiments. As shown in Fig. 2, 2'-OH RNA-AS (SEQ ID NO:2) inhibited TS mRNA translation better than DNA-AS (SEQ ID NO:1), however modified antisense oligonucleotides, HYB0432, and HYB0431, inhibited TS mRNA translation to an even greater extent than the unmodified 2'-OH RNA-AS.

In a third study inhibition of human and *E. coli* TS mRNA translation with HYB0431 and HYB0432 was compared. Human TS mRNA (100ng) and *E. coli* TS mRNA (300ng) in combination with either HYB0431 or HYB0432 were incubated in

rabbit reticulocyte lysate reactions. The results (not shown) indicate that both HYB0432 (2'-Me RNA-AS) and HYB0431 (2'-OMe RNA-AS phosphorothioate) specifically inhibited TS mRNA translation. At concentrations much higher (greater than 10 fold) than that required for inhibitory effects to be shown, HYB0431 demonstrated non-specific effects by inhibiting translation of control mRNA (*E. coli* TS).

In a fourth study, specific repression of human TS mRNA translation by HYB0432 was studied. Human TS mRNA, *E. coli* TS mRNA, dihydrofolate reductase (DHFR) mRNA, Xenopus elongation factor (Xef) mRNA, luciferase mRNA and human p53 mRNA were separately incubated in RRL in the presence of absence of a 50-fold molar excess of HYB0432. The results (not shown) indicate that HYB0432 inhibits human TS mRNA in a highly specific manner.

In yet another series of experiments, the ability of antisense oligonucleotides of the invention to inhibit growth in human cancer cells was studied. Inhibition of human breast cancer MCF-7 cells by HYB0432 antisense oligonucleotide was tested by incubating MCF-7 cells with lipofectin (20 ug/ml) containing HYB0432 for 24 hours. Cells were then allowed to grow for an additional 48 hours in fresh medium after which time the cells were counted. The results are shown in FIG. 3. HYB0432 in combination with lipofectin inhibited growth of MCF-7 cells in a concentration dependent manner.

In order to determine whether antisense oligonucleotides of the invention complementary to TS nucleic acid are able to control TS expression, cells treated with such oligonucleotides were analyzed for TS protein. TS protein levels as well as control tubulin protein levels were examined by Western blot in human MCF-7 cells following treatment for 24 hours with TS-specific oligonucleotide concentrations ranging from 0.1  $\mu$ m to 1.0  $\mu$ m. Western blot analysis was performed using an anti-TS monoclonal antibody and an anti-alpha tubulin monoclonal antibody. As shown in FIG. 4, levels of TS protein were decreased in a concentration-dependent manner following treatment with the HYB0432 (SEQ ID NO:2) oligonucleotide. A maximum inhibition of about 70% of TS was observed as compared to no inhibition of tubulin protein.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

## EXAMPLES

### 1. Synthesis and Analysis of Oligonucleotides

Oligonucleotides of the invention and control oligonucleotides were synthesized, purified, and analyzed using standard procedures as are known in the art.

Phosphorothioate deoxynucleosides are synthesized on CPG on a 5-6  $\mu$ mole scale on an automated synthesizer (model 8700, Millipore, Bedford, MA) using the H-phosphonate approach described in U.S. Patent No. 5,149,798. Deoxynucleoside H-phosphonates are obtained from Millipore (Bedford, MA). 2'-O-methyl ribonucleotide H-phosphonates or phosphorothioates are synthesized by standard procedures (see, e.g., "Protocols for Oligonucleotides and Analogs" in Meth. Mol. Biol. (1993) Vol. 20) or commercially obtained (e.g., from Glenn Research, Sterling, VA and Clontech, Palo Alto, CA). Segments of oligonucleotides containing 2'-O-methyl nucleoside(s) are assembled by using 2'-O-methyl ribonucleoside H-phosphonates or phosphorothioates for the desired cycles. Similarly, segments of oligonucleotides containing deoxyribonucleosides are assembled by using deoxynucleoside H-phosphonates for the desired cycles. After assembly, CPG bound oligonucleotide H-phosphonate is oxidized with sulfur to generate the phosphorothioate linkage. Oligonucleotides are then deprotected in concentrated  $\text{NH}_4\text{OH}$  at 40°C for 48 hours.

Crude oligonucleotide (about 500  $\text{A}_{260}$  units) are analyzed on reverse low pressure chromatography on a  $\text{C}_{18}$  reversed phase medium. The DMT group is removed by treatment with 80% aqueous acetic acid, then the oligonucleotides are dialyzed against distilled water and lyophilized.

All 2'-OMe oligoribonucleotide phosphorothiates and phosphates were synthesized in 1 umol scale of solid support on EXPITIDE (PerSeptive Biosystems) using *tert*-butylphenoxyacetyl (t-PBA) protected phosphoramidites in each synthetic cycle. The oxidation step was done by either using Beaucage reagent or iodine in pyridine and water. To completely deprotect and release oligonucleotides, support-bound oligoribonucleotide was treated with 28%  $\text{NH}_4\text{OH}$  at 55°C for 12 hours. Purification and separation of oligo were done on a preparative  $\text{C}_{18}$  HPLC column. After detritylation with 80% acetic acid and dialysis, final oligo solution was lyophilized to a white powder and oligos were analyzed by CGE and  $^{31}\text{P}$  NMR.

## EXAMPLE 2

## IN VITRO TRANSLATION STUDIES

The rabbit reticulocyte lysate translation system was purchased from Promega. Briefly, mRNAs were added to the reticulocyte lysate mix containing  $^{35}$ S-labeled methionine and the RNase inhibitor, Inhibitase (5prime, 3 Prime, Inc.). Reactions were incubated for 1 hour at 30°C after which time 2 volumes of Laemmli sample buffer were added to the reaction mix followed by a 5 min. incubation at 65°C. Denatured lysates were resolved by SDS/PAGE (15% acrylamide). Gels were processed according to previously published methods (Chu et. al., *PNAS* 88:8977 (1991)). Quantitation of signal intensities was performed by densitometric scanning.

## EXAMPLE 3

## GROWTH INHIBITION STUDIES

Human breast cancer MCF-7 cells were plated at a density of  $5 \times 10^4$  cells/well. Cationic liposomes (Lipofectin) containing HYB0432 were prepared according to the Gibco-BRL procedure. After 24 hours the liposome-containing medium was removed and fresh RPMI 1640 medium (10% dialyzed FBS) was added. After an additional 48 hours, cells were trypsinized and counted.

EXAMPLE 4  
WESTERN IMMUNOBLOT ANALYSIS

MCF-7 cells were plated at a density of  $2 \times 10^5$  cells/well. Liposomes in the presence of HYB0432 were prepared and added to the wells. After 48 hours of incubation at 37°C cells were removed by scraping and lysed by sonication. Cell extracts were resolved by SDS/PAGE (15% acrylamide). Gels were processed according to previously published methods (Chu et al., *PNAS* 88:8977 (1991)).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

What is claimed is:

1. A synthetic oligonucleotide complementary to a TS nucleic acid.
2. The synthetic oligonucleotide of claim 1 which is complementary to a portion of TS nucleic acid which encodes the 5' untranslated region, the 3' untranslated region, the translational start site, the translational stop site, the cis acting regulatory element in the 5' upstream region, or a splice junction site.
3. The synthetic oligonucleotide of claim 1 consisting essentially of a nucleic acid sequence selected from the group consisting of SEQ ID NOS:1, and 2.
4. The oligonucleotide of claim 1 having 6 to 50 nucleic acids.
5. The oligonucleotide of claim 4 having 15 to 30 nucleic acids.
6. The oligonucleotide of claim 5 having 20 to 25 nucleic acids.
7. The oligonucleotide of claim 1 which is modified.
8. The modified oligonucleotide of claim 7 comprising an internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamide, and carboxymethyl esters.
9. The oligonucleotide of claim 8 comprising at least one phosphorothioate internucleotide linkage.
10. The oligonucleotide of claim 8 comprising phosphorothioate internucleotide linkages.
11. The oligonucleotide of claim 1 comprising at least one deoxyribonucleotide.
12. The oligonucleotide of claim 1 comprising at least one ribonucleotide.

13. The oligonucleotide of claim 12 wherein the ribonucleotide is a 2'-O-substituted ribonucleotide.
14. The oligonucleotide of claim 13 wherein the ribonucleotide is a 2'-O-alkylated ribonucleotide.
15. The oligonucleotide of claim 14 wherein the ribonucleotide is a 2'-O-methylated ribonucleotide.
16. The oligonucleotide of claim 11 further comprising at least one ribonucleotide.
17. The oligonucleotide of claim 16 comprising at least one 3'-terminal 2'-O-methylated ribonucleotide.
18. The oligonucleotide of claim 16 comprising at least one 5'-terminal 2'-O-methylated ribonucleotide.
19. The oligonucleotide of claim 17 further comprising at least one 5'-terminal 2'-O-methylated ribonucleotide.
19. The oligonucleotide of claim 17 comprising at least two 5'-terminal 2'-O-methylated ribonucleotides and at least two 3'- terminal 2'-O-methylated ribonucleotides.
20. The oligonucleotide of claim 19 having modified internucleotide linkages.
21. The oligonucleotide of claim 20 having phosphorothioate internucleotide linkages.
22. The oligonucleotide of claim 1 which is capable of downregulating at least a portion of TS mRNA translation and/or TS protein expression.
23. A method of downregulating at least a portion of TS mRNA translation or TS protein expression in a malignant cell, comprising the step of administering to the cell an oligonucleotide of claim 1 in an amount sufficient to specifically downregulate at least a portion of TS mRNA translation and/or TS protein expression in the cell.

24. A method of preventing or overcoming resistance to TS inhibitor compounds in a malignant cell, comprising the step of administering to the cell an amount of at least one oligonucleotide of claim 1 sufficient to downregulate at least a portion of TS mRNA translation and/or TS protein expression in a cell.
25. A therapeutic composition comprising at least one oligonucleotide of claim 1 and a pharmaceutically acceptable carrier or diluent.
26. A method of treating malignant tumor growth in a mammal comprising the step of administering to the mammal the therapeutic formulation of claim 25 in an amount sufficient to reduce tumor growth.
27. The oligonucleotide of claim 12 comprising all ribonucleotides.
28. The oligonucleotide of claim 27 wherein all ribonucleotides are 2'-OMe substituted ribonucleotides.
29. The oligonucleotide of claim 28 wherein all internucleotide linkages are phosphorothioate internucleotide linkages.

## Translational Inhibition of T<sub>4</sub> mRNA by Antisense RNA Oligo

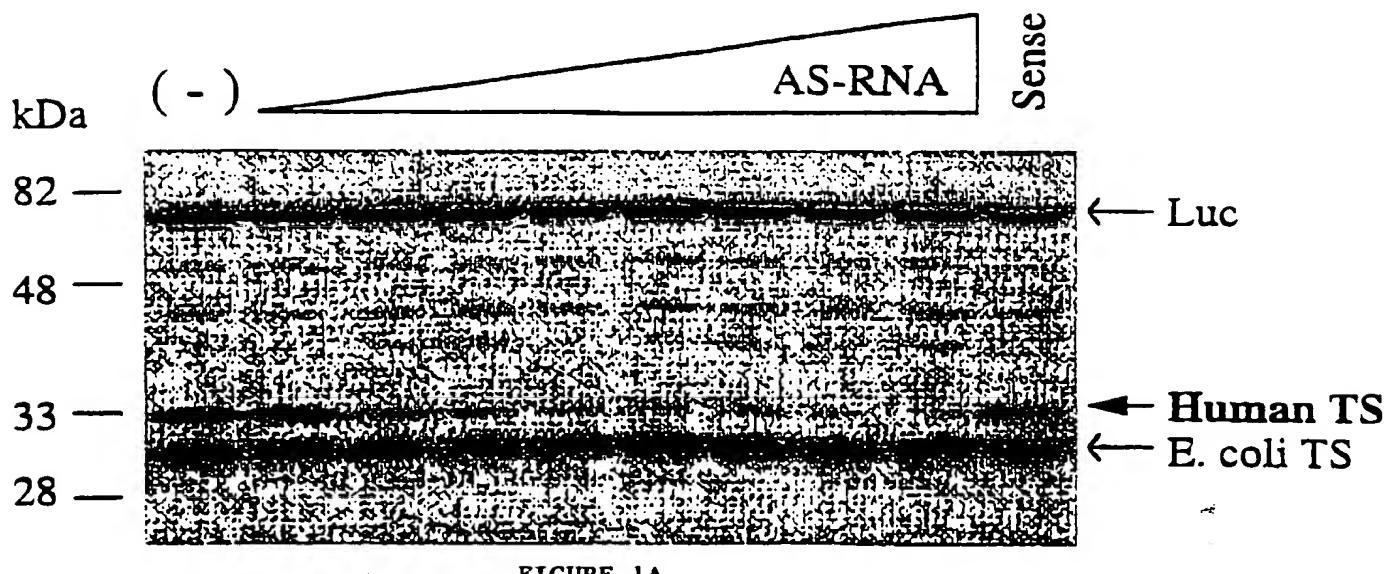


FIGURE 1A

### Representative Densitometry

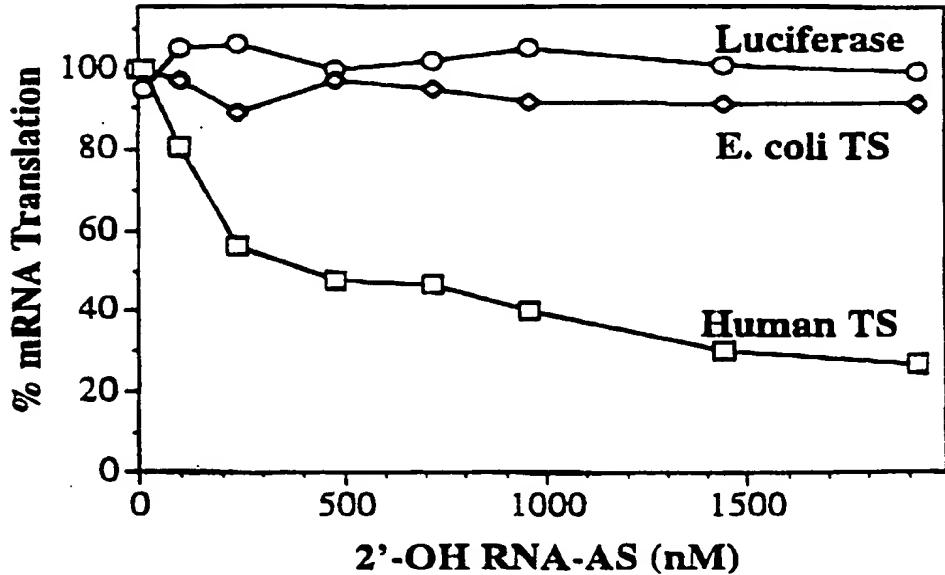
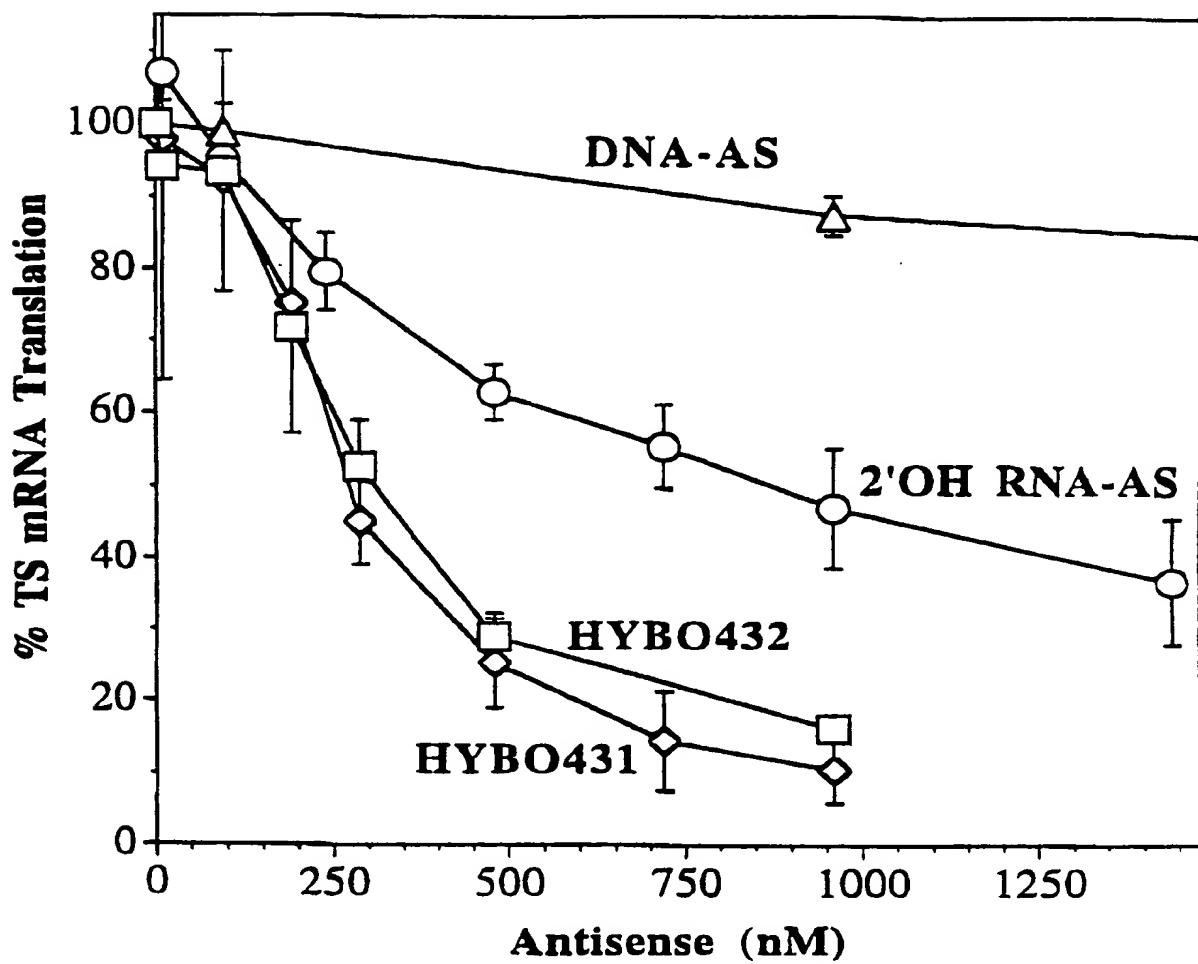


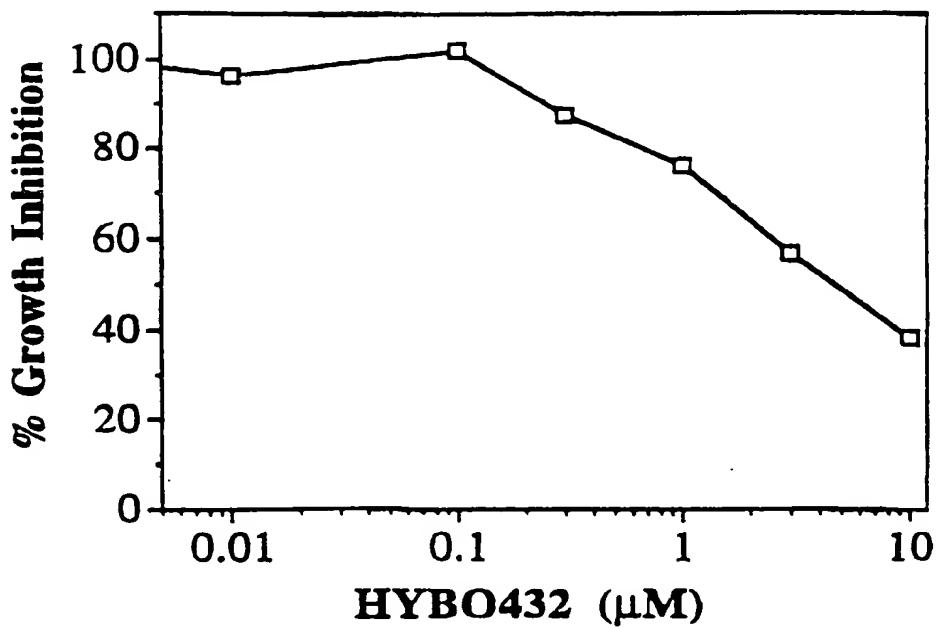
FIGURE 1B

**Inhibition of TS mRNA Translation by Modified Antisense RNAs**



**FIG. 2**

**Growth Inhibition of Human Breast Cancer MCF-7 Cells by  
HYBO432**



**FIG. 3**

## Western Analysis of TS Protein in MCF-7 Cells after Treatment with HYBO432

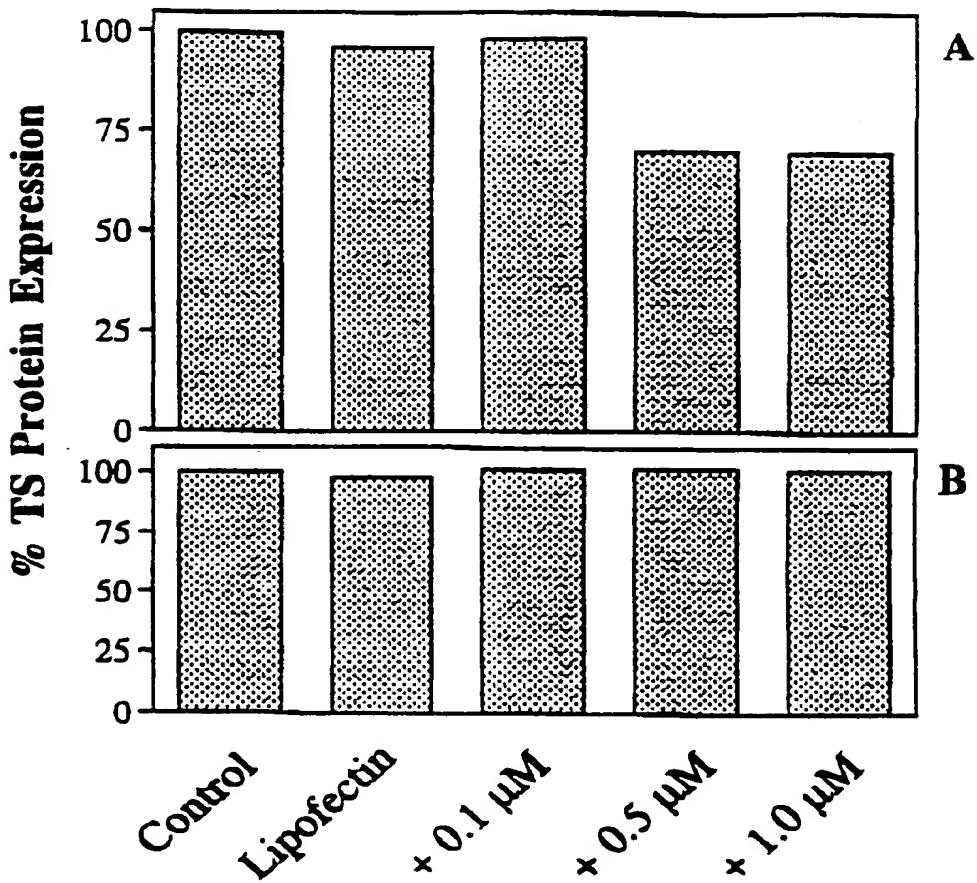


FIGURE 4A

B

FIGURE 4B

FIG. 5





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/11, A61K 31/70, C07H 21/00</b>		A3	(11) International Publication Number: <b>WO 98/49287</b> (43) International Publication Date: 5 November 1998 (05.11.98)
<p>(21) International Application Number: <b>PCT/US98/08722</b></p> <p>(22) International Filing Date: 30 April 1998 (30.04.98)</p> <p>(30) Priority Data: 60/045,230 30 April 1997 (30.04.97) US</p> <p>(71) Applicant: HYBRIDON, INC. [US/US]; 620 Memorial Drive, Cambridge, MA 02139 (US).</p> <p>(72) Inventors: SCHMITZ, John, C.; Apartment 2, 81 Edwards Street, New Haven, CT 06511 (US). AGRAWAL, Sudhir; 61 Lamplighter Drive, Shrewsbury, MA 01545 (US). CHU, Edward; 346 Saybrook Road, Orange, CT 06477 (US).</p> <p>(74) Agents: KERNER, Ann-Louise et al.; Hale and Dorr LLP, 60 State Street, Boston, MA 02109 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report.</i></p> <p>(88) Date of publication of the international search report: 4 February 1999 (04.02.99)</p>	
<p><b>(54) Title:</b> ANTISENSE OLIGONUCLEOTIDES SPECIFIC FOR THYMIDYLATE SYNTHASE</p> <p><b>(57) Abstract</b></p> <p>The present invention discloses synthetic oligonucleotides complementary to thymidylate synthase and methods of downregulating at least a portion of thymidylate synthase mRNA translation or protein expression in a malignant cell and of preventing or overcoming resistance to thymidylate synthase inhibitor compounds in a malignant cell using such oligonucleotides. Also disclosed is a therapeutic composition and a method of treating malignant tumor growth in a mammal utilizing such a composition.</p>			

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/08722

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 6 C12N15/11 A61K31/70 C07H21/00

According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JU, JINGFANG : "INCREASED DRUG SENSITIVITY OF TUMOR CELLS BY MANIPULATION OF CELL CYCLE CONTROL GENES" PH.D. THESIS PRESENTED IN DECEMBER 1996 AT THE FACULTY OF THE GRADUATE SCHOOL UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES, XP002081365 Y see page 100 - page 130 --- Y WO 94 02498 A (WORCESTER FOUND EX BIOLOGY ;METELEV VALERI (US); AGRAWAL SUDHIR (U) 3 February 1994 see the whole document --- -/-	1-5, 11, 22-26  7-21, 27-29  7-21, 27-29  -/-

Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

20 October 1998

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHU E ET AL: "New concepts for the development and use of antifolates." STEM CELLS, (1996 JAN) 14 (1) 41-6., XP002081366 see page 44, left-hand column, last paragraph - right-hand column, line 18; figure 3 ---	1, 2, 12, 24, 27
X	KOBAYASHI H ET AL: "Reversal of drug-resistance or inhibition of tumor cell growth by hammerhead ribozymes;" GENE THER.;(1995) 2, PAGE 689; ABSTRACT 065, XP002081367 see paragraph 3 ---	1, 2, 4, 12, 22-24, 27
X	WO 93 13740 A (WORCESTER FOUND EX BIOLOGY) 22 July 1993 see page 10 - page 11 see page 14, line 4 - line 11 see page 15, line 19 - page 16, line 20 see page 23: table 1 ---	1, 4-19, 22, 23
X	KOROPATNICK J ET AL: "Discrimination of run-on transcription from constitutive genes and antisense expression vectors in the same cells." BIOTECHNIQUES;(JAN 1997) 22, 1, 64-66, XP002081368 see the whole document ---	1-5, 12, 27
A	LIU Y ET AL: "Levamisole potentiation of 5-fluorouracil and other anti-tumour drugs in cultured cells: Protein tyrosine phosphatase as an alternative to thymidylate synthase as a target." CELLULAR PHARMACOLOGY 2 (6). 1995. 271-277., XP002081369 see the whole document ---	1-29
A	CHU E ET AL: "Identification of an RNA binding site for human thymidylate synthase." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1993 JAN 15) 90 (2) 517-21., XP002081370 cited in the application ---	
A	CHU E ET AL: "Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1991 OCT 15) 88 (20) 8977-81., XP002081371 cited in the application ---	

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BLACK A R ET AL: "Expression of rTS correlates with altered growth regulation of thymidylate synthase." CANCER RESEARCH, (1996 FEB 15) 56 (4) 700-5., XP002081372 ---	
P, X	SCHMITZ J C ET AL: "Specific repression of human TS mRNA translation by antisense oligoribonucleotides." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING 39 (0). MARCH 1998. PAGE 645; ABSTRACT 4391, XP002081373 see abstract & 89TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, NEW ORLEANS, LOUISIANA, USA, MARCH 28-APRIL 1, 1998. , -----	1-29

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/08722

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 23 and 24 (as far as in vivo methods are concerned) and claim 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
The application contains twice claim 19. All dependent claims have been searched as being dependent on both claims 19.
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No

PCT/US 98/08722

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9402498	A 03-02-1994	US 5652355	A	29-07-1997	
		AU 674158	B	12-12-1996	
		AU 4780193	A	14-02-1994	
		CZ 9500143	A	17-07-1996	
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WO 9313740	A 22-07-1993	CA 2126979	A	22-07-1993	
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